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DEVELOPMENT OF LOW PHYTATE PEAS

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FINAL REPORT

"Development of Low Phytate Peas"

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(a) Summary

Phytate commonly forms one to several percent of the dry weight of seeds, and in many cases accounts for 50-80% of the total phosphorus in seeds. Phytate is generally not digested by humans and non-ruminant livestock; instead most is excreted. As a result, the substantial phosphorus stores in grains are essentially wasted. The objective of this project was to develop field pea lines in which the phosphorus is primarily stored in the inorganic (digestible) form, rather than in the phytate form, i.e., "low phytate peas". Chemical mutagenesis was used in an attempt to induce low phytate mutations in pea seeds. A similar approach has been used in corn, soybean, and barley.

This study identified at least 25 low phytate field pea lines derived from cultivar CDC Bronco using colorimetric and HPLC assays. Phytate levels in these lines ranged from 22-77% of those found in CDC Bronco. These lines were developed using non-GMO techniques.

(b) Executive Summary

Historically, pulse crops have typically been used in human consumption diets around the world. However, in the case of field pea, both food and feed markets are important. Field pea is a good feed ingredient, especially for monogastric animals, such as pigs and poultry, due to its high lysine content, high energy value, and low level of antinutritional compounds.

Phosphorus, phytic acid and phytate are found in significant amounts in crop seeds and fruits. Phytate is a mixed cation salt of phytic acid (*myo*-inositol hexa*kis* phosphoric acid). Phytic acid is a powerful chelator. Phytate commonly forms one to several percent of the dry weight of many seeds, and in many cases

accounts for 50-80% of the total P in seeds. Despite its role in plant nutrition, several factors contribute to the 'phytic acid problem'. Phytate is generally not digested by humans and non-ruminant livestock; most is excreted. As a result, the substantial P stores in grains are essentially wasted. Important micronutrients (such as Zn^{++} and Fe^{+++}) bound to phytate are also excreted, potentially leading to micronutrient deficiencies. In addition, P-containing effluents have been linked to eutrophication of lakes and waterways in Europe and North America.

Chemically induced, non-lethal recessive mutants that reduce seed phytic acid content have recently been isolated and genetically mapped in maize, barley, and rice. These low phytic acid mutations have the potential to alleviate the nutritional and environmental problems associated with phytic acid in animal feeds. These mutants show large effects in the partitioning of P into phytic acid P and inorganic P; in that phytic acid P reductions are counterbalanced increases in inorganic P which is readily digestible by humans and non-ruminant livestock.

Low phytate field pea varieties will increase the value of crop production because they will be a higher value feed ingredient, they will reduce the costs of livestock feed supplementation by reducing the need for addition of inorganic-P and/or phytase, and they will enhance environmental stewardship by reducing the concentration of phosphorus in manure effluents. **Thus, the objective of this project was to develop low phytate pea mutants.**

Field pea cultivar CDC Bronco was selected for development of low phytate lines via mutagenesis. Seed of CDC Bronco was exposed to the chemical mutagens sodium azide or ethyl methane sulfonate (EMS) by Dr. V. Raboy (USDA). The treated seed was planted in an off-season nursery in Arizona in November 2003 and M1 seeds were bulk harvested in April 2004. We harvested approximately 80,000 in total. Approximately 7000 M1 seeds were planted in Saskatoon in summer 2004 in a space-planted nursery, while the remaining seeds were held in reserve. Approximately 2500 single plants were harvested in September 2004 from each of the sodium azide and EMS treatments. In 2005, single seeds from each of the harvested plants were tested for the low phytate trait using a colorimetric assay. Putative low phytate mutants were re-tested by HPLC.

This study identified at least 25 low phytate field pea lines derived from cultivar CDC Bronco. Phytate levels in these lines ranged from 22-77% of those found in CDC Bronco. These lines were developed using non-GMO techniques.

Phase 2 of this research should involve characterization of these low phytate mutants at the chemical and genetic levels. The long term goal is the development of low phytate field pea varieties. These varieties would provide a low phytate protein source to complement a low phytate energy source from barley, soon to be released from Dr Rossnagel's program at the Crop Development Centre.

In the future, this research will provide a low phytate protein feed source to complement a low phytate energy source from barley, soon to be released from Dr. Rossnagel's program at the Crop Development Centre.

(c) Technical Report

Introduction

Pulse crop production has expanded dramatically in western Canada in recent years. The five year average (2000-2004) area of field pea production in western Canada is 1.23 million hectares with average production of 2.34 million tonnes (Agriculture and Agri-Food Canada, 2004). Area of production of pulse crops in western Canada has increased because pulse crops have generally been more profitable for producers than cereals and oilseeds. In addition, pulse crops provide nitrogen and non-nitrogen benefits to cereal crops in the rotation.

Historically, pulse crops have typically been used in human consumption diets around the world. This is the key end-use of Canadian lentil, chickpea, dry bean, and fababean production. In the case of field pea, both food and feed markets are important. The five year average (1998-99 to 2002-03 crop years) utilization of Canadian field pea production was estimated at 1.01 million tonnes for feed, 0.96 million tonnes for food, and 0.24 million tonnes for seed (Stan Strypetz, Agriculture and Agri-Food Canada, personal communication). Field pea is a good feed ingredient, especially for monogastric animals, such as pigs and poultry, due to its high lysine content, high energy value, and low level of antinutritional compounds. Over the past 15 years, the largest user of Canadian peas for feed has been feed compounding companies in western Europe. The five year average (1998-99 to 2002-03 crop years) use of field pea in domestic animal rations has increased to account for approximately one-third of the portion of Canadian pea production used for feed (Stan Strypetz, Agriculture and Agri-Food Canada, personal communication). This proportion is expected to continue to increase as area of production increases, and as the livestock industry expands.

Phosphorus, phytic acid and phytate are found in significant amounts in crop seeds and fruits. Phytate is a mixed cation salt of phytic acid (*myo*-inositol hexa*kis* phosphoric acid). Negatively charged sites of phytic acid bind mainly K^{+} and Mg^{++} but also form salts with other cations including Ca^{++}, Mn^{++}, Zn^{++}, or Fe^{+++}. Phytic acid is a powerful chelator. Phytate commonly forms one to several percent of the dry weight of many seeds, and in many cases accounts for 50-80% of the total P in seeds. Lott et al. (2000) estimated the following levels (%) of phytic acid in pulse crops: pea (1.00), lentil (0.70), chickpea (0.55), dry bean (1.41), and fababean (1.11). The functions of phytate in seeds and seedlings are thought to include storage of inositol, P, K, Mg, Ca, Mn, Fe and Zn; and control of inorganic P levels in developing seeds and seedlings (Lott et al., 2000).

Despite its role in plant nutrition, several factors contribute to the 'phytic acid problem'. Phytate is generally not digested by humans and non-ruminant livestock; most is excreted. As a result, the substantial P stores in grains are essentially wasted. Important micronutrients (such as Zn^{++} and Fe^{+++}) bound to phytate are also excreted, potentially leading to micronutrient deficiencies. In addition, P-containing effluents have been linked to eutrophication of lakes and waterways in Europe and North America.

Chemically induced, non-lethal recessive mutants that reduce seed phytic acid content have recently been isolated and genetically mapped in maize, barley, and rice (Larson et al., 2000). These low phytic acid mutations have the potential to alleviate the nutritional and environmental problems associated with phytic acid in animal feeds (Ertl et al., 1998). These mutants show large effects in the partitioning of P into phytic acid P and inorganic P; in that phytic acid P reductions are counterbalanced by molar-equivalent increases in inorganic P. Inorganic P is readily digestible by humans and non-ruminant livestock. These low phytic acid mutants typically show 50-75% reductions in phytic acid P, with no reduction in total P in the seed, and little or no effect on plant and seed growth and function (Lott et al., 2000). Initial field studies indicated that the first round of low phytic acid cultivars performed adequately with yield reductions between 0% to approximately 15% compared to non-mutant controls.

We have conducted preliminary studies to evaluate the phosphorus, phytate, and inorganic-P levels in a set of six field pea varieties from the CDC breeding program which were grown at two locations in 2002. Results showed that the proportion of phosphorus in the phytate form ranged from 50-60%, similar to that of other crops assayed.

Following chemical mutagenesis, low phytate mutants were detected at a frequency of approximately 1 in 200 plants assayed in barley, 1 in 1000 plants assayed in corn, and 1 in 3000 assayed in soybean (Raboy, personal communication). Thus, we expect to find low phytate mutants in field pea at a frequency within the above range.

Low phytate field pea varieties will increase the value of crop production because they will be a higher value feed ingredient, they will reduce the costs of livestock feed supplementation by reducing the need for addition of inorganic-P and/or phytase, and they will enhance environmental stewardship by reducing the concentration of phosphorus in manure effluents.

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Research Progress

Objective: The objective of this project was to develop low phytate pea mutants.

Hypothesis to be tested: Low phytate pea mutants can be developed and identified.

Materials and Methods:
Preliminary analyses were conducted in Dr. Raboy's lab to evaluate the range in variation in phosphorus, phytate, and inorganic-P levels in a set of six field pea varieties grown at two locations in 2002 in Saskatchewan. Results showed that the proportion of phosphorus in the phytate form ranged from 50-60%, similar to that of other crops assayed.

One of the six varieties, CDC Bronco, was selected for development of low phytate lines via mutagenesis. This variety was selected based on its superior agronomic performance in field trials in western Canada in recent years. In addition, CDC Bronco had relatively high protein content and low acid detergent fibre content in wet chemistry analyses conducted on samples harvested in 2001 (annual report to ADF on project #20000229).

Two kg of seed of CDC Bronco was divided into four lots and these were exposed to the chemical mutagens sodium azide (1 mM) for either 3 or 4 hours, or ethyl methane sulfonate (EMS) 0.75% or 1.5% for four hours. The treated seed was planted in an off-season nursery in Arizona in November 2003 and M1 seeds were bulk harvested in April 2004. Approximately 80,000 plants were harvested in total. A minor deviation occurred when the mutagenized seeds were planted in the winter nursery in that the two sodium azide treatments were planted into one plot instead of two, and the two EMS treatments were planted into one plot instead of two. Thus, from then on the total number of mutagen treatments was reduced to two (sodium azide and EMS) from four. This deviation did not substantially affect the progress of this research project.

Approximately 7000 M1 seeds were planted in Saskatoon in summer 2004 in a space-planted nursery, while the remaining seeds are being held in reserve. Approximately 2500 single plants were harvested in September 2004 from each of the sodium azide and EMS treatments for a total of 5000 plants. These plants were threshed individually in spring 2005. We obtained approximately 50-100 seeds per plant .

Several apparent mutant plants were observed in the 2004 nursery including plants deficient in chlorophyll expression, powdery mildew susceptible plants, Fusarium wilt susceptible plants, and one plant with abnormal leaf structure.

In summer 2005, single seeds were tested for the low phytate trait using a colorimetric assay (Raboy, 1997) under the supervision of Dr. Gene Arganosa. Drs. Arganosa and Rossnagel have been routinely conducting this assay on barley samples for the past several years. Briefly, 117 plants X 3 seeds per plant plus 3 controls were assayed per set consisting of four 96-well micro-titre plates. Individual kernels were crushed into 2.0 ml microcentrifuge tubes. Five hundred ul of 0.4 M hydrochloric acid was added to each tube after which the tubes were stored overnight at 4°C. Tubes were then vortexed for 10 s, and allowed to settle for 30 min. Ten ul of extract and phosphorus standard were assayed using a modification of Chen's method (Chen et al. 1956). For the sample extracts, 10 ul of the extract was pipetted into a microtiter plate well after which 90 ul of double distilled water was added to bring the total volume in each well to 100 ul. For the P standards, 0, 10, 20, 30, 40 and 50 ul of 1 mM K_2HPO_4 were pipetted into separate wells after which 90, 80, 70, 60, 50 and 40 ul of double distilled water were added, respectively. Finally, 10 ul of 0.4 M HCl was also added to bring the total volume in each well to 100 ul.

Chen's reagent was prepared daily and consists of 1 volume 6N H_2SO_4, 1 volume 2.5% ammonium molybdate, 1 volume 10% ascorbic acid and 2 volumes of double distilled water (Chen et al., 1956). One hundred ul was added to each well and the mixtures were allowed to stand for 2 hr before reading the absorbance values on a microplate plate (655 nm filter). A standard curve is calculated based on the concentration of the standards and their absorbance values. The absorbance value for each well is converted into ul of 1 mM K_2HPO_4.

Seeds with normal phytate levels produced colourless solutions, while low phytate seeds produced blue solutions depending on the amount of phytate reduction. The degree of phytate reduction was quantified using a plate reader. We assayed approximately 300 plants per week, thus, requiring approximately 4 months to analyse 5000 plants. Putative low phytate mutants were re-tested using the same assay in fall 2005, and then by HPLC using a technique in routine use in the Department of Animal and Poultry Science, University of Saskatchewan.

Samples are prepared for HPLC analyses by weighing 0.15 g in duplicate into labeled screw-capped 2 ml flat-bottomed high speed centrifuge tubes after which 1.5 ml 0.5 N hydrochloric acid was added. Tubes were vortexed well and then shaken in black racks for 3 hours on an orbital shaker at 300 rpm at ambient temperature. Tubes were spun on a microfuge for 10 min. The supernatant from each tube was transferred with a pasteur pipette into separate 12 x 75 mm glass tubes. Two hundred fifty ml of chloroform was added with a pasteur pipet. Tubes were vortexed and spun on a benchtop centrifuge at 3,000 rpm for 10 min. The top layers were transferred to clean, labelled HPLC vials and frozen until ready for injection.

A phytate stock was prepared by dissolving 0.0310 g phytate (Sigma, 62% pure) in 2 ml 0.5 N HCl or 0.0248 g phytate (Sigma, 80.2% pure) in 2 ml 0.5 N HCl. Twenty-five, 50, 75, 100, 125 and 150 ul of phytate stock were diluted with 975, 950, 925, 900, 875 and 850 ul of 0.5 N HCl, respectively to make a final volume of 1000 ul.

The inositol di- through hexaphosphates in both the phytate standards and sample solutions were measured using an HPLC anion exchange method with a post-column reagent for detection (Newkirk and Classen, 1998). Inositol phosphates were determined using an HPLC System Gold (Beckman Instruments, Mississuaga, ON, Canada) equipped with a Hamilton 50 x 4.6 mm PRP X-500 column and a 6.3 x 11.2 mm pre-column (Hamilton, Reno, NV, U.S.A.). Samples (100 ul) were eluted over 20 min gradient (0 to 0.5 M NaNO3, 0.01 M 1-methylpiperzine, pH 4.0) and a flow rate of 1.5 ml/min. The post-column reagent (0.015% FeCl3.6H20 (w/v), 0.15% (w/v) sulfosalicylic acid) was also delivered by a Perkin Elmer (Vancouver, BC, Canada) Model 250 pump at 1.5 ml/min at an absorbance of 500 nm. Peak areas were quantified from the standard curve for inositol phosphate as described above.

References

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Newkirk, R.W. and Classen, H.L. 1998. In vitro hydrolysis of phytate in canola meal with purified and crude sources of phytase. Animal Feed Science and Technology 72:315-327.

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Results and Discussion

Colorimetric assay

Normal barley seeds (variety CDC McGwire), low phytate barley seeds (variety HB 378) and normal CDC Bronco seeds were used as checks in the colorimetric assays. The normal barley seeds and normal CDC Bronco seeds typically had

3-8 units (ul of 1 mM K_2HPO_4), while the low phytate barley seeds typically had 25-30 units. The low phytate barley check variety (HB378) is known to have phytate levels that are reduced by approximately 50% in comparison to normal barley. The vast majority of the field pea lines derived from this research had phytate values in the range of 3-8 units. We assumed these were non-mutant for the phytate trait. However, approximately 50 lines were identified that had phytate values >10 units. These were retested with the colorimetric assay and in most cases results were consistent. These lines were considered as putative low phytate pea mutants.

HPLC assay

Initially, the ten pea lines which appeared to have the greatest phytate reduction were evaluated by the HPLC assay. CDC Bronco seeds had phytate levels of 0.74% (Table 1). In comparison, nine of the ten CDC Bronco derived pea lines assessed had reduced phytate levels (0.34-0.57%), while one appeared to have normal phytate levels. Thus, these nine lines had phytate levels which were 44-77% of normal.

In a second HPLC study, a comparison was made between pea lines that differed widely in terms of phytate values in the colorimetric assay. Ten lines with apparently normal phytate levels (color values ranging from 1.3-4.5) from each mutagenesis treatment were compared with ten lines with apparently reduced phytate levels (color values ranging from 8.6-12.1) from each mutagenesis treatment (Table 2). Of the apparently normal phytate lines, 16 of 20 had phytate levels between 0.62-0.88% as determined by HPLC, i.e., similar to that of CDC Bronco (0.78%), while four lines had phytate levels between 0.50-0.56% suggesting some phytate reduction. In contrast, 16 of 20 of the apparently low phytate lines had phytate levels between 0.22-0.52% as determined by HPLC, while four lines had higher levels. Thus, these 16 lines had phytate levels which were 28-67% of normal.

A third HPLC study, conducted following a similar protocol as the second study, is in progress.

Thus, based on the combined results of the two HPLC studies, this research has identified 25 low phytate pea lines, expressing a range of phytate levels from 28-77% of CDC Bronco, the variety from which they were derived.

Future directions

This project was planned as the initial phase of a larger project to develop low phytate field pea cultivars for use in commercial feed and food applications. Phase 2 in this research should involve characterization of the low phytate mutants generated in this project using genetic and biochemical studies including the following.

-Grow out plots of the low phytate lines in the field in 2006 and assess phytate levels of single plants. Controls will include CDC Bronco, normal phytate CDC Bronco sub-lines, normal barley, low phytate barley.

-Cross selected low phytate lines with normal phytate pea lines and evaluate inheritance of the low phytate trait in P1, P2, F1, F2, BCF1, BCF2, F3 generations.

-Develop recombinant inbred lines (RILs) from one or more of the above crosses for the purpose of mapping and marking the low phytate gene(s).

-Increase seed of a few low phytate pea lines for use in feeding studies in poultry and pigs in diets containing normal feed components and low phytate feed components such as low phytate barley variety HB 378.

In the future, this research will provide a low phytate protein source to complement a low phytate energy source from barley, soon to be released from Dr. Rossnagel's program at the Crop Development Centre.

This project proceeded on schedule with no substantial deviations from the original work plan.

Acknowledgments. The advice of Dr. B. Rossnagel and Dr. V. Raboy in designing and interpreting this research project is gratefully acknowledged.

Papers/Presentations:
None thus far.

Information of benefit to producers, processors, or governments:
In the future, this research will provide a low phytate protein source to complement a low phytate energy source from barley, soon to be released from Dr. Rossnagel's program at the Crop Development Centre.

(d) Personnel:
Technical assistance from Arnulfo Lima was charged to this project.

(e) Equipment purchased:
None

(f) Project Developed Materials:
Several low phytate pea lines were produced.

(g) Project Photos:
None thus far.

(h) Acknowledgement:

The Saskatchewan Agricultural Development Fund and the Saskatchewan Pulse Growers were acknowledged in all presentations where this research project was mentioned.

(i)Expense Statement:
Will be provided.

(j) ICAR Data Entry
Will be entered.

Table 1. Evaluation of phytate levels in ten putative low phytate pea lines in comparison to normal phytate pea and barley varieties and a low phytate barley variety.

Sample ID	Mean Color Value	Phytate (IP6, % as is).
CDC McGwire (normal phytate barley)	8.5	1.10
HB 378 (low phytate barley)	27.3	0.49
CDC Bronco (normal phytate pea)	6.1	0.74
Trt 1-536	9.6	0.57
Trt 1-1576	12.0	0.34
Trt 1-2347	18.4	0.39
Trt 2-56	10.5	0.50
Trt 2-114	11.3	0.38
Trt 2-1019	9.9	0.57
Trt 2-1036	10.0	0.87
Trt 2-1237	10.4	0.57
Trt 2-1468	9.8	0.38
Trt 2-1531	10.4	0.38

Note: Pea lines labelled as 'Trt 1-' were derived from sodium azide mutagenesis, while those labelled as 'Trt 2-' were derived from ethyl methane sulfonate (EMS) mutagenesis.

Table 2. Evaluation of phytate levels in 20 putative low phytate pea lines in comparison to 20 putative normal phytate pea lines, a normal and a low phytate barley variety, and a normal phytate pea variety.

Sample ID	Mean Color Value	Phytate (IP6, % as is)		Mean Color Value	Phytate (IP6, % as is)
Normal Phytate					
Trt 1-4	1.3	0.62	Trt 2-497	4.2	0.88
Trt 1-5	3.7	0.50	Trt 2-892	4.5	0.65
Trt 1-1799	3.9	0.85	Trt 2-1552	4.5	0.62
Trt 1-1861	3.6	0.83	Trt 2-1723	4.5	0.54
Trt 1-2389	3.8	0.67	Trt 2-2138	3.4	0.85
Trt 1-2393	3.8	0.82	Trt 2-2149	4.4	0.74
Trt 1-2412	2.7	0.77	Trt 2-2343	4.2	0.76
Trt 1-2621	2.8	0.71	Trt 2-2393	3.7	0.73
Trt 1-2623	3.6	0.56	Trt 2-2461	4.2	0.69
Trt 1-2628	3.1	0.74	Trt 2-2477	3.8	0.53
Low Phytate					
Trt 1-29	12.1	0.47	Trt 2-5	8.6	0.26
Trt 1-121	10.4	0.68	Trt 2-40	10.3	0.27
Trt 1-150	9.1	0.22	Trt 2-41	9.0	0.45
Trt 1-208	9.3	0.52	Trt 2-99	9.0	0.63
Trt 1-330	9.6	0.90	Trt 2-325	9.2	0.39
Trt 1-372	10.8	0.45	Trt 2-600	9.5	0.39
Trt 1-451	9.2	0.49	Trt 2-947	9.4	0.30
Trt 1-744	9.1	0.34	Trt 2-1259	8.6	0.49
Trt 1-1070	9.3	0.48	Trt 2-1269	8.6	0.86
Trt 1-1077	10.6	0.37	Trt 2-1417	8.7	0.49
Checks					
CDC McGwire	8.5	0.93			
HB 378	27.3	0.47			
CDC Bronco	6.1	0.78			

Note: Pea lines labelled as 'Trt 1-' were derived from sodium azide mutagenesis, while those labelled as 'Trt 2-' were derived from ethyl methane sulfonate (EMS) mutagenesis.